

# History of U.S. Military Contributions to the Study of Bacterial Zoonoses

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Bacterial zoonoses have afflicted campaigns throughout military history, at times playing an important role in determining their outcomes. In addition, zoonotic bacteria are among the leading biological warfare threats. The U.S. military medical services have been at the forefront of research to define the basic microbiology, ecology, epidemiology, and clinical aspects of these diseases. This historical review discusses the military significance of plague, Q fever, anthrax, leptospirosis, bartonellosis, tularemia, and brucellosis and the U.S. military medical research counteroffensive. These contributions have ranged from basic molecular biology to elegant epidemiological surveys, from defining pathogenesis to developing new vaccine candidates. In an era of emerging diseases and biological weapons, the U.S. military will continue to lead a dynamic research effort to counter these disease threats.

## Introduction

Bacterial zoonoses have afflicted military campaigns throughout history, at times playing an important role in determining their outcomes. In addition, because of their stability in aerosols, infectivity following inhalation, and virulence, several zoonotic pathogens are among the most formidable biological weapon threats. The U.S. military medical services have been at the forefront of research to define the basic microbiological, ecological, epidemiological, and clinical aspects of these diseases.

## Plague

One of the most notorious episodes in the military history of bacterial zoonoses ended the siege of Caffa in 1346. Caffa was a Genoese trading center on the Crimean coast, under attack by the Tatar forces under Janibeg, Khan of the Golden Horde. After a year of siege, the defenders were holding out behind their heavily fortified walls, showing no signs of defeat. Meanwhile, an epidemic of plague decimated the Tatar forces, resulting in their abandoning the attack. Before they withdrew, they launched one last parting shot. They catapulted "mountains of dead" into the city, in the contemporary account of Gabriel de Mussis, "in the hope that the intolerable stench would kill everyone inside." This was followed by an epidemic in which "the defenders died widely" of a clinical syndrome consistent with bubonic plague; this appears to have been a tactically successful biological attack.<sup>1,2</sup>

Both the Tatar camp and Caffa might have been prone to rat infestation and epizoonotic disease because of poor sanitation and crowding. The pivotal question is whether *Yersinia pestis* was introduced into Caffa by rodent reservoirs, imported cases, or hurled cadavers. Importation by a rodent-flea-human transmission cycle is biologically plausible. Plague is thought to have been spreading westward along the central Asian caravan routes into Crimea, transported to cities by rats aboard ships or other vehicles or possibly by fleas present on hides in the fur trade.<sup>3,4</sup> Although land routes to Caffa were severed, according to historical sources, maritime trade was maintained during the siege.<sup>1</sup> This would provide a portal of entry for rat-borne plague or imported cases. An alternative hypothesis would be importation of plague from the Tatar camp to Caffa by sylvatic rodents.

*Y. pestis* transitioned from urban to sylvatic rodent reservoirs in the United States early in the last century. Although the spread of plague from sylvatic back to urban rodents is biologically plausible and was evidenced by the isolation of *Y. pestis* from a dead rat and two dead squirrels in Dallas, Texas, in 1993, this appears to be infrequent under current ecological conditions.<sup>5,6</sup> U.S. military researchers identified *Y. pestis* infection in bandicoots (*Bandicota indica*), members of a sylvatic rodent species trapped in urban plague foci in Vietnam, but stopped short of implicating bandicoots in the geographic expansion of *Y. pestis* over long distances. It remains unclear whether the bandicoots contracted *Y. pestis* from rats or vice versa.<sup>7</sup>

The Caffa epidemic was revisited by Dr. Mark Wheelis of the University of California.<sup>1</sup> The Tatar front lines were probably several hundred meters away from the walls of Caffa, out of range of the defender's weapons. Their main encampment was probably even farther back, to provide more security and space for logistical support. Rats are sedentary and rarely venture far from their nests; therefore, it is unlikely that they would have covered an open area of several hundred meters. Furthermore, the Centers for Disease Control and Prevention reported that up to 20% of cases of plague contracted in the United States result from direct contact with body fluids or tissues of infected animals or inhalation of droplets or other infectious aerosols.<sup>6</sup> If the defenders were handling thousands of cadavers (and probably splatter generated on impact), they might well have contracted plague from the attack. Dr. Wheelis concluded that transmission from contact with the cadavers is the most biologically plausible of several hypotheses, based on rodent ecology and disease transmission.

## U.S. Military Significance

The first large-scale U.S. deployment to a plague-endemic area occurred during World War II in the China-India-Burma theater. The U.S. military strategy of using the Haffkine inactivated whole-cell vaccine is especially interesting in historical

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context. Occasional Haffkine vaccine failures and the introduction of live attenuated plague vaccines in the early 1930s led to a virtual abandonment of inactivated plague vaccines; the value of the Haffkine vaccine was seriously questioned by the start of World War II.<sup>8</sup> The live vaccines have since been characterized as pigmentation-deficient strains, because of the spontaneous deletion of a 102-kb chromosomal fragment encoding iron-binding and -transporting functions.<sup>9</sup> They are more immunogenic than killed vaccines in animals but are highly reactogenic. K.F. Meyer evaluated dosing regimens and reactogenicity of the inactivated vaccine at military hospitals and concluded that the vaccine was less reactogenic than the inactivated whole-cell typhoid vaccine in use in 1940. There were no cases of plague among vaccinated U.S. troops during World War II.<sup>10</sup> Evaluation of three distinct whole-cell vaccine formulations continued into the 1970s and led to a dosing strategy that reduced local and systemic reactions by 70% and 65%, respectively.<sup>11,12</sup>

### U.S. Military Contributions

Vietnam emerged as the world's leading country in plague incidence during the war years of the 1960s. Military researchers led by such luminaries as Lieutenant Colonel John D. Marshall, Jr., Dr. Dan C. Cavanaugh, and their colleagues at the Walter Reed Army Institute of Research (WRAIR), the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), and Naval research and public health units elegantly defined the geographic distribution and demographic features of the epidemic and expanded our knowledge of the basic microbiology, reservoir and vector biology, ecology, clinical aspects, and epidemiology of plague.<sup>10,13-15</sup> They described multifocal, urban, rat-borne epidemics fueled by the movement of more than 6 million refugees from farmland into urban slums.<sup>15</sup> Dr. Marshall and colleagues determined that the twin cities of Saigon and Cholon remained enzootic plague foci throughout the year, with infected animals trapped in five well-defined foci. In addition, infected rodents and fleas were present in and around U.S. military facilities. The investigators broke new ground by noting that, because of a large number of infected fleas, control of both rodents and vectors would be essential for effective plague control.<sup>13</sup>

All U.S. troops entering Vietnam received the inactivated vaccine. There were only eight cases, at a rate hundreds of times less than that among the Vietnamese. Dr. Marshall suggested a number of explanations, including differences in living conditions, rodent and vector control, protective clothing, repellants, and use of the Haffkine vaccine.<sup>10</sup> Evidence for subclinical *Y. pestis* infections among vaccinated U.S. troops was sought in serological studies of patients with murine typhus, because the two diseases are transmitted by the same flea vector (*Xenopsylla cheopis*). Acute and convalescent sera from 58 soldiers with serologically confirmed murine typhus were studied for antibody against the fraction 1 (F1) antigen of *Y. pestis*. Sixty-seven percent of patients were seropositive at baseline, reflecting vaccination status. Convalescent testing 7 to 14 days later disclosed a fourfold or greater increase in titer for 7% and seroconversion for 5%, suggesting that 12% of murine typhus patients had subclinical *Y. pestis* coinfection, with clinical plague being averted by vaccination. This gave indirect evidence of the efficacy of the Haffkine vaccine. In addition, the incidence rates of plague among Vietnamese civilians and of murine typhus

among U.S. troops were correlated with a seasonal expansion of the common flea vector and increased rodent flea density during the spring. This linked climate, vector ecology, and plague transmission.<sup>10</sup>

Dr. Cavanaugh related ambient temperature to the activity of specific enzymes of *Y. pestis* and the flea vector. He described the expression of *Y. pestis* coagulase at 25°C, leading to coagulation of the blood meal and obstruction of the flea gut, and the expression of a *Y. pestis* fibrinolytic factor and a flea-gut trypsin-like enzyme at 37°C, potentially contributing to clot lysis and decreased gut flea blocking at higher temperatures. These observations explain the phenomenon of gut flea blocking and possibly the observed seasonal variations in transmission, with lower rates occurring in hot weather.<sup>16</sup>

The historical conventional wisdom was that the antiphagocytic F1 capsular glycoprotein was an essential virulence factor.<sup>15</sup> F1 is expressed at low levels at temperatures below 27°C (e.g., in the flea gut). Plague bacilli infect the host, are phagocytized by macrophages and transported to regional lymph nodes, replicate intracellularly, and express F1 at 37°C. The macrophages lyse and release bacilli with the antiphagocytic F1 antigen into the bloodstream.

We are indebted to COL A.M. Friedlander and colleagues in the USAMRIID Bacteriology Division for clarifying that the F1 antigen is not an essential virulence factor. They demonstrated that F1-negative strains were virulent in laboratory animals, with essentially no difference in virulence between F1-positive and F1-negative strains in a nonhuman primate aerosol-challenge model.<sup>17</sup> This is especially interesting in the biological defense context. An adversary could potentially use an F1-deficient strain to defeat F1-based detection and diagnostic assays. Furthermore, even if an F1-positive strain were used, F1 would not be expressed if the bacilli were incubated and stored at low ambient environmental temperatures (e.g., in a munition or in a spray tank) under many climatic conditions, because expression is temperature dependent; F1-based detection systems would then be ineffective. Other molecular targets for vaccines, diagnostic assays, and detection kits offer more potential utility than F1-based systems.

An in vitro study demonstrated that treating *Y. pestis* with rabbit polyclonal or monoclonal antibodies directed against the V antigen protected a murine macrophage cell line against *Y. pestis*-induced apoptosis and also reversed the inhibition of phagocytosis. This supports the role of V antigen as a virulence factor and as a potential vaccine component.<sup>18</sup>

The low efficacy of the whole-cell vaccine in protecting laboratory animals against inhalation plague has implications for biological warfare defense and has spurred interest in new vaccine candidates. Dr. D.G. Heath and colleagues in the USAMRIID Bacteriology Division evaluated a subunit vaccine using a recombinant F1-V fusion protein. The vaccine protected mice against experimental pneumonic plague from both F1-positive and F1-negative strains.<sup>19</sup>

We may be heading back to the future with a live attenuated plague vaccine. Dr. S.L. Welkos and colleagues at the USAMRIID compared the virulence of three *Y. pestis* mutant strains, i.e., a pigment-deficient strain, a plasminogen activator-deficient strain, and a double-mutant strain lacking both virulence factors. The double mutant was the most attenuated, with a mouse

50% lethal dose ( $LD_{50}$ ) 1 to 3 log units greater than that of other strains. Surviving mice also withstood subsequent lethal wild-type strain challenges. Monkeys survived aerosol challenges and generated significant antibody titers. This suggests that the double mutant may have a role as a new attenuated vaccine, potentially less reactogenic than the live vaccines developed in the 1930s and still used in parts of the developing world.<sup>20</sup>

COL W.R. Byrne, Dr. Welkos, and colleagues at the USAMRIID compared the efficacy of five antibiotic classes for the treatment of inhalational plague in mice after an aerosol challenge of 100  $LD_{50}$  units of *Y. pestis*. Ciprofloxacin and ofloxacin were comparable to the aminoglycosides.  $\beta$ -Lactams, aztreonam, and rifampin were not effective, in contrast to their in vitro activity.<sup>21</sup>

## Q Fever

No summary of military contributions to the sciences of infectious disease and public health would be complete without homage to luminary A.S. 'Bud' Benenson. Before serving 25 years as editor of the Control of Communicable Diseases Manual of the American Public Health Association,<sup>22</sup> COL Benenson contributed to numerous endeavors in the course of his U.S. Army career, including the development of the first effective Q fever vaccine.

Q fever (Query fever, also known as Queensland fever), caused by *Coxiella burnetii*, is a disease with unusual variability in duration and severity. Transmission can be direct (by aerosol or ingestion) or by tick vector. Most human cases are acquired by inhaling infectious agent where infected animals are kept, particularly dairy barns and lambing sheds, or consuming unpasteurized milk. Infection may be unapparent or present as a nonspecific fever of unknown origin, resulting in an incapacitating illness of 2- to 3-week duration. Pneumonitis with "ground glass" appearance may be found on roentgenograms, but productive cough, chest pain, and physical findings in the lungs are not prominent. Elevated liver function levels are common. In chronic form, Q fever may describe an indolent course with endocarditis manifest on morphologically abnormal or prosthetic cardiac valves. Laboratory diagnosis is usually through acute/convalescent-stage antibody immunofluorescence, complement-fixation, enzyme-linked immunosorbent, or microagglutination assays. Isolation of the pathogen from blood is diagnostic but presents hazards to laboratory workers. Q fever *coxiellae* may also be identified in tissue (liver biopsy or heart valve) by immunostaining and electron microscopy. The organism has two antigenic phases, i.e., Phase I, found in nature, and Phase II, observed after multiple laboratory passages in eggs or cell culture. The organism is unusually resilient, can reach high concentrations in animal tissues, particularly placenta, and is highly resistant to many disinfectants. *C. burnetii* is extremely infectious for humans, with a single viable organism being sufficient to cause infection; there is concern regarding potential biowarfare applications of this agent.<sup>23</sup>

## U.S. Military Significance

Epidemiological considerations suggest that Q fever was a significant infectious disease factor in the American Civil War. The scorecard on major illness during that conflict is numbing

in its enormity. Among Union soldiers, pneumonia (including influenza and bronchitis) accounted for 1,765,000 episodes of illness and 45,000 deaths. Although pneumonic illness was the most commonly reported, diagnostic criteria of the time would probably have subsumed some cases of Q fever under "other miasmatic disease, not classified."<sup>24</sup> Therefore, it probably contributed to the failure of several important Union campaigns early in the war, prolonging the war if not affecting the ultimate outcome. The impact on Confederate forces might have been disproportionately greater because of their more rural demographic profile, but no data exist because Confederate medical records were systematically burned by victorious Union forces at the end of the war.

The agent of Q fever was isolated and the entity established as a tick-borne disease in 1937.<sup>25,26</sup> The disease was originally thought to occur only in Australia but its cosmopolitan nature was quickly established, with military investigators in World War II retrospectively diagnosing the disease among British and U.S. troops in Italy and Greece.<sup>27</sup> The rubric under which it was diagnosed then was primary atypical pneumonia. The contemporary moniker for the disease among British forces in Greece was "Balkan grippé." Suspecting a transmissible febrile disease, guinea pig blood was sent to the Pasteur Institute in Athens by the U.S. Army Epidemiological Board Commission on Acute Respiratory Diseases. Underscoring the personal risk attendant to such seminal research, a laboratory outbreak caused by this "Balkan grippé" strain occurred among personnel of the Fort Bragg, North Carolina-based commission before the agent was characterized in test material sent from Greece. Wartime investigations of Q fever carried out by members of U.S. Army medical units considerably extended clinical knowledge of the disease, particularly with respect to the frequency of pulmonary involvement, and two widely separated geographic areas were added to its known distribution, specifically, the northern Mediterranean region and Panama. Concomitant German reporting suggests outbreaks among Axis troops in Italy, Bulgaria, Greece, the Ukraine, and the Crimea.<sup>28</sup>

Seven outbreaks among U.S. forces in the Mediterranean theater during World War II were confirmed by serology and animal inoculation and resulted in approximately 1000 cases. Cases were epidemiologically linked to occupation of barns and two-story dwellings, using the top floor as an apartment for human use and the ground floor as a barn.<sup>27,29</sup>

During May 1945, an outbreak of an acute febrile illness occurred among troops returning by ship from Italy. During the first 36 hours ashore, 62 patients were hospitalized. Eighty-nine percent were from the 717th Bomber Squadron. The squadron was placed under quarantine, and another 78 squadron members were hospitalized over the next 2 weeks, for an attack rate of 38%. Q fever was implicated by serological testing.<sup>29</sup> The squadron had been stationed at the Grottaglie Air Base. The troops were billeted in renovated military buildings or tents. None of the buildings had been used as barns, there was no livestock on the base, and there was no direct contact between the troops and livestock. However, the pastures adjacent to the base were used for goat and sheep herding. Bomber operations stopped in the last week of April. On May 12, the squadron members departed by aircraft for Naples. The investigating epidemiologists did not identify a source of the epidemic. They

would not implicate environmental aerosols generated by aircraft because an incubation period of 10 to 14 days would not associate aerosols generated by bombers in late April with an epidemic occurring in late May. However, the cases fell within 40 days of the final bomber operations in the last week of April,<sup>29</sup> within a currently accepted incubation period of 10 to 40 days (Fig. 1). Another hypothesis is generated by reviewing the epidemic curve using an incubation period of 1 to 4 weeks.<sup>30</sup> Most cases occurred 7 or more days after departure from Grottaglie. An untestable hypothesis would be that perhaps the members had the bad luck to be standing on the flight line, waiting for the plane that would take them to Naples, and were exposed to an aerosol of *C. burnetii* generated by propeller draft on departure. Cases occurred in the other four squadrons at Grottaglie, for an estimated attack rate of 30% among the 1,400 troops stationed there.<sup>29</sup>

After World War II, serodiagnostics were used to confirm Q fever infections throughout the United States as well as around the world. An explosive outbreak among U.S. military personnel stationed in Libya was characterized by U.S. Air Force CAPT Francis Fellers.<sup>31</sup> The disease was implicated in an outbreak of 50,000 cases in California in 1948,<sup>32</sup> and Navy LT Philip Snodgrass reported endemic Q fever around a Naval Air Station in south Texas in 1956.<sup>33</sup> Sporadic cases of Q fever occurred during the 1950s and among troops during Operations Desert Storm, Restore Hope,<sup>34</sup> Enduring Freedom, and Iraqi Freedom. An unusual case of a patient returning from Operation Desert Storm with meningoencephalitis and transient focal neurological deficits, suggesting transient ischemic attacks, was reported from Wilford Hall Medical Center.<sup>35</sup>

Q fever was diagnosed at four U.N. Stabilization Force bases in Bosnia and Herzegovina in 1997. This outbreak primarily afflicted Czech soldiers. A sheep farm with active lambing was located 100 m from the most-affected base, with an approach path for helicopter operations generating aerosols. Environmental aerosols were considered the most likely source of the epi-

demic; this was supported by anecdotal reports of a flu-like syndrome among sheep workers on the adjacent farm.<sup>36</sup>

### U.S. Military Contributions

U.S. military research contributions included studies of the aerobiology of *C. burnetii*, inoculum effects, clinical aspects, and vaccine development.<sup>34</sup> A 1 million-liter aerosolization chamber known as the "8 Ball" was used at Fort Detrick during the 1950s and 1960s for animal and human volunteer challenge studies.<sup>37,38</sup> The investigational inactivated vaccine prepared from *C. burnetii* Phase I-infected yolk sac, pioneered by U.S. military physicians, may be useful for protecting laboratory workers and others knowingly working with live *C. burnetii*; it should also be considered for deployed troops at high risk and for abattoir workers. Vaccine may be obtained under an Investigational New Drug protocol by contacting the commanding officer, U.S. Army Medical Research and Materiel Command, Fort Detrick (Frederick, Maryland). Because of a problematic side effect profile, this vaccine is not commercially available in the United States (another Q fever vaccine is licensed in Australia). The vaccine is reactogenic among previously immune individuals; this requires a cumbersome two-step process of skin-testing vaccine candidates with a small dose of vaccine and then excluding reactors from vaccination. Contraindications to vaccination include a positive skin or antibody test or a documented history of Q fever. Consequently, a major goal is a less-reactogenic Q fever vaccine. Dr. D.M. Waag and colleagues at the USAMRIID used a chloroform-methanol extraction process to develop a Phase I antigen subunit vaccine candidate that is safe and immunogenic among unscreened human volunteers and that protects mice and guinea pigs against aerosol challenge.<sup>39,40</sup>

### Anthrax

*Bacillus anthracis* has long been considered a leading biological warfare threat. U.S. military researchers have made leading

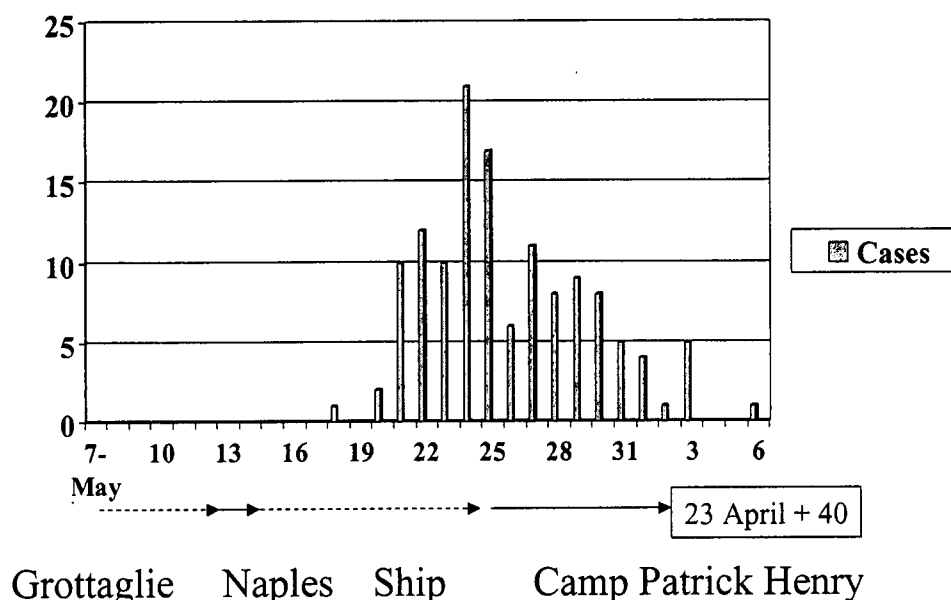


Fig. 1. Incidence of Q fever in the 717th Bomber Squadron, May to June 1945. Cases occurred within the 10- to 40-day incubation period after cessation of bomber operations during the last week of April, suggesting exposure to environmental aerosols because of propeller draft.

contributions regarding anthrax. These include studies of the genetic and molecular mechanisms of virulence and immunity, animal models of pathogenesis, studies of pre-exposure and postexposure immunoprophylaxis, chemoprophylaxis, and treatment, development of recombinantly produced, protective antigen (PA) vaccine candidates, and development of diagnostic reagents.

The currently licensed anthrax vaccine was developed during the 1950s and 1960s, when the three principal protein virulence factors of *B. anthracis* were identified, e.g., PA, lethal factor (LF), and edema factor (EF).<sup>41,42</sup> Tissue edema and necrosis result from EF and/or LF, which act as cytopathic (A) chains of anthrax toxins according to the A-B model of dichain toxins.<sup>43</sup> PA serves as the carrier protein (B chain) for both EF and LF and binds to receptors on target cell membranes to initiate endocytosis. All three toxin component proteins are encoded on a single plasmid, pXO1. The current vaccine uses PA as an immunogen, resulting in the generation of anti-PA antibodies.

### U.S. Military Contributions

USAMRIID researchers demonstrated that nonhuman primates could withstand aerosol challenges up to 950 LD<sub>50</sub> units of inhaled *B. anthracis* spores given 6 to 100 weeks after vaccination.<sup>44-46</sup> A study of the effects of immediate postexposure prophylaxis with either chemoprophylaxis alone or vaccination with and without chemoprophylaxis disclosed that chemoprophylaxis was protective during a 4-week course but 10% to 30% of unvaccinated animals died of anthrax after the prophylaxis was stopped, apparently because of survival of inhaled spores. The combination of vaccination and chemoprophylaxis was protective; chemoprophylaxis afforded time for the development of postvaccination immunity.<sup>47</sup>

USAMRIID scientists have investigated the potential use of recombinant technology to produce highly purified PA, eliminating the risk of trace contamination with LF or EF during standard vaccine production. The gene encoding PA has been cloned into *Bacillus subtilis* using a recombinant plasmid.<sup>48</sup> In addition, *B. anthracis* strains cured of the pXO1 plasmid have been transformed by inserting recombinant plasmids that encode PA. In addition to their potential in the manufacture of purified PA, these strains may represent a future generation of live attenuated vaccines, potentially less reactogenic than those used in the former Soviet Union and in veterinary medicine, which express all three toxin components. These vaccine candidates have protected guinea pigs against aerosol challenge.<sup>48,49</sup>

Various adjuvants have been studied. The combination of purified PA with monophosphoryl lipid A was more effective than the standard aluminum adjuvant PA vaccine in protecting guinea pigs against an aerosol spore challenge. Moreover, lyophilization had no effect on vaccine potency, a potential advantage over the current vaccine, which requires cold storage.<sup>50</sup>

Dr. Welkos and colleagues at the USAMRIID Bacteriology Division demonstrated that the protection afforded by anti-PA antibodies might be attributable to mechanisms other than toxin neutralization. Cell cultures of mouse macrophages were infected with spores that had been opsonized with immune sera from rabbits vaccinated with either standard or recombinantly produced PA; preimmune serum; or culture medium. Phagocytosis was enhanced by sera containing anti-PA antibodies. There was no significant effect of anti-PA antibodies on phago-

cytosis when the study was repeated using spores from a recombinant pXO1-deficient strain. This suggested that PA or PA-like proteins are expressed on the spore surface and may be molecular targets for anti-PA antibodies. In addition, incubation of spores in sera containing anti-PA antibodies inhibited germination, as measured by both changes in optical density of the culture media and percentages of germinated spores determined by direct microscopy. Spore-associated proteins recognized by anti-PA antibodies were detected by electron microscopy and confirmed by immunoblot analysis and gel electrophoresis of spore coat proteins. This series of experiments suggested that PA is expressed on the spore surface and that anti-PA antibodies serve as opsonins and interfere with early stages of infection, before the elaboration of toxin by vegetative bacteria.<sup>51</sup>

Challenges facing us as a military force have included the cumbersome six-dose vaccination schedule and concerns over local reactions. COL P.R. Pittman and colleagues at the USAMRIID Medical Division completed a randomized, open-label, pilot study to select an optimal, two-dose, initial vaccination schedule and route of administration, based on antibody responses and reactogenicity.<sup>52</sup> IgG antibody titers were measured among volunteers who received either the standard subcutaneous injections at 0 and 2 or 4 weeks, intramuscular injections at 0 and 4 weeks, or subcutaneous injections at 1 and 4 weeks. With the exception of a greater initial increase in titers in the standard dosing group, there were no significant differences in titers during the first 6 months. Local reactions were more common among female subjects, regardless of the route of administration, but were significantly less frequent following intramuscular injection for both genders. A larger study is underway to confirm these results.

### Leptospirosis

Leptospirosis is a bacterial zoonosis of worldwide distribution that is caused by spirochetes of the genus *Leptospira*. Disease can range from mild flu-like illness to life-threatening pulmonary hemorrhage or Weil's disease. Infection occurs more commonly in tropical climates, typically after contact with freshwater contaminated with urine from colonized rodents.

### U.S. Military Significance

Epidemics of leptospirosis occurred widely during World War I, likely because of trench warfare crowding, rat infestation, poor drainage, standing water, and mud. The etiology was determined independently by British, French, and German medical services. Although trench warfare was obsolete during World War II, epidemics of leptospirosis were identified on both sides and were epidemiologically linked to bathing in streams and rivers. The British also identified prolonged contact with mud in an area in France as a risk factor. Only 30 cases among U.S. troops were diagnosed, all in the Pacific and Burma-India theaters. The low reported incidence possibly reflected difficulty in clinical diagnosis; this is underscored by the delayed identification of an epidemic febrile exanthem at Fort Bragg.<sup>53</sup>

During the summer of 1942, an outbreak of an illness featuring fever, malaise, lumbar pain, severe frontal headache, and a distinctive cutaneous eruption occurred among 40 troops at

Fort Bragg. The rash featured irregular erythematous lesions of 2- to 5-cm diameter that gradually coalesced. The pretibial surfaces were the only site of involvement in 60% of cases and the primary site of involvement in an additional 20%. Diffuse involvement was noted in 5% in cases; only 12.5% had no rash. Other findings included splenomegaly and leukopenia. The etiological agent was not identified, although an environmental reservoir was suggested by the geographic localization of the epidemic to an area of the installation containing a small stream and its tributaries.<sup>54</sup> Outbreaks recurred in the summers of 1943 and 1944.<sup>53</sup> The etiological agent of "Fort Bragg fever" was identified by Maj W.S. Gochenour and colleagues at the Army Medical Service Graduate School (the precursor of the WRAIR) in 1951-1952, following isolation of *Leptospira autumnalis* after multiple animal passages of the "Fort Bragg agent" and agglutination tests using sera from case patients and human volunteers and animals infected during serial passages and large collections of leptospiral strains.<sup>55</sup> Also in 1952, Maj Gochenour and colleagues confirmed leptospirosis as the cause of epidemic aseptic meningitis among U.S. service members in Okinawa in 1949.<sup>56</sup>

Leptospirosis was a major disease of U.S. military members during the Vietnam conflict.<sup>57</sup> In a 2-year study at a field hospital in northern South Vietnam, leptospirosis was the leading cause of febrile illness undiagnosed after the first 2 days of hospitalization, accounting for 20% of such cases.<sup>58</sup> Outbreaks among U.S. military personnel occurred in Panama in 1961, Okinawa in 1987, and Oahu in 1992; these epidemics underscored the association of leptospirosis with recreational freshwater swimming and contact with standing water during military training.<sup>59-62</sup>

### U.S. Military Contributions

During two field exercises in Panama in the early 1980s, 2% to 8% of U.S. troops contracted leptospirosis. Dr. E.T. Takafuji and colleagues at the WRAIR demonstrated that prophylaxis using doxycycline (200 mg once per week) conferred a 95% risk reduction.<sup>63</sup> The utility of antibiotic therapy for leptospirosis was controversial into the 1980s. U.S. military researchers demonstrated the benefits of doxycycline for treatment of anicteric leptospirosis and penicillin for treatment of late disease in randomized, placebo-controlled, double-blind trials.<sup>64,65</sup> More recently, Drs. C.K. Murray and D.R. Hoshenthal at Brooke Army Medical Center refined broth microdilution susceptibility testing for leptospira<sup>66</sup> and studied the susceptibilities of seven species to more than 30 antimicrobial agents. Macrolide and ketolide agents had the best in vitro activity. The expanded-spectrum cephalosporins, including cefepime, cefotaxime, and ceftriaxone, and fluoroquinolones had excellent activity. The activity of ampicillin was enhanced by the addition of sulbactam; whether this was attributable to the elaboration of  $\beta$ -lactamase by leptospires or an antibiotic effect of sulbactam is not clear.<sup>67</sup> The identification of *Leptospira* and malaria coinfections raised the possibility of using antimalarial agents to treat both diseases. However, artemisinin, atovaquone, chloroquine, mefloquine, primaquine, proguanil, pyrimethamine, sulfadoxine, quinine, quinidine, and atovaquone/proguanil and pyrimethamine/sulfadoxine combinations did not have significant in vitro activity.<sup>68</sup>

### Bartonellosis

U.S. military medical personnel have made vast contributions to our understanding of the basic bacteriology, ecology, epidemiology, and clinical aspects of zoonoses caused by the genus *Bartonella*. Increasingly recognized as the cause of human disease, *Bartonella* species have been identified as the cause of clinical syndromes ranging from cat scratch disease (CSD) to visceral peliosis, neuroretinitis, endocarditis, and Oroya fever. Many other clinical syndromes are caused by these bacteria, and an increasing array of *Bartonella* species have been implicated as the source of human illness.

Of the zoonotic *Bartonella* species, *Bartonella henselae* is the most frequently encountered and causes a wide spectrum of disease in immunocompetent and immunocompromised hosts. U.S. military scientists have played a significant role in the understanding of this pathogen, its clinical syndromes, and treatment. Rarely encountered as human pathogens, other zoonotic *Bartonella* species such as *Bartonella vinsonii* are beyond the scope of this review.

Two species of *Bartonella* causing a significant human disease burden have no known reservoir other than their human hosts. The first identified member of the genus, *Bartonella bacilliformis*, does not inhabit a known animal reservoir in its life cycle, although many authors have suspected this possibility. Infection causes a biphasic illness in residents and visitors in regions of the South American Andes. One *Bartonella* species has resulted in more human disease than the others combined. Trench fever, the result of infection by *Bartonella quintana*, was responsible for enormous morbidity and death during wars in the early 20th century and earlier. Also called typhus fever, this disease is transmitted from person to person by human body lice, without an intermediate host. Although military researchers have been responsible directly and indirectly for the identification of these pathogens, their epidemiology, clinical manifestations, and treatment, they are not considered in this review of zoonoses.

Shortly after the initial identification of a novel, fastidious, Gram-negative bacillus causing prolonged fever among immunocompetent and immunocompromised persons,<sup>69</sup> *Rochalimaea henselae*, which was later reclassified into the genus *Bartonella*, was identified in two Air Force patients who had sustained tick bites.<sup>70</sup> These individuals developed febrile illnesses that relapsed repeatedly following multiple, short-course, antibiotic treatments. In both cases, *B. henselae* was isolated from the blood using novel culture techniques. Growth characteristics as well as polymerase chain reaction amplification and sequencing conclusively identified the organism as *B. henselae*. This study served to demonstrate a new clinical syndrome associated with this infection (aseptic meningitis), identified a potential arthropod vector (tick), and showed the possibility of prolonged bacteremia similar to that seen after infection with *B. bacilliformis* and requiring prolonged antibiotic therapy for clearance. Cultivation of the organism remains challenging and was a major barrier to identifying its role as an etiological agent. The improvement in culture methods demonstrated in this study aided investigators broadly and led directly to improved understanding of *B. henselae* as a human pathogen.

Early investigations demonstrated *B. henselae* as a cause of bacillary angiomatosis and peliosis hepatitis among patients



infected with human immunodeficiency virus (HIV).<sup>71</sup> Following the described associations with fever and bacteremia among both immunocompromised and immunocompetent patients, as well as aseptic meningitis and relapsing febrile illness,<sup>69,70</sup> military investigators sought to explore the relationship between *Bartonella* and CSD. Previously, researchers at the Armed Forces Institute of Pathology had isolated a small, Gram-negative bacillus from lymph node tissue from 10 patients with CSD, which was later designated *Afpia felis*.<sup>72</sup> The role of *A. felis* as the sole cause of CSD was called into question as a new serological test for *B. henselae* was developed and used by the Centers for Disease Control and Prevention to study this disease. Patients with clinical CSD had high antibody titers against *B. henselae* but not *A. felis*.<sup>73</sup>

Using an improved culture method, Air Force investigators made the first recovery of *B. henselae* from lymph nodes of two patients with clinical CSD. Multiple techniques, including whole-cell fatty acid analysis and citrate synthase gene sequencing, were used to confirm the organism as *B. henselae*.<sup>74</sup>

This study was one of the cornerstones establishing the role of *B. henselae* as the leading etiological agent of syndromic CSD and served to expand the understanding of this disease. In addition, culture methods were refined and led to development of a chemically defined liquid medium for isolation of *B. henselae*.<sup>75</sup> Later studies by Dr. D.M. Demers and colleagues at Tripler Army Medical Center provided further evidence of *B. henselae* as the predominant etiological agent of CSD, with no evidence of *A. felis* infection in their series.<sup>76</sup> The epidemiological link between *B. henselae* infection risk and exposure to young cats was further validated and again arthropod exposure (ticks and/or fleas) was suggested as a potential means of transmission. Further epidemiological work has supported the notion that cat fleas may play a significant role in transmission of *B. henselae* between cats and humans.<sup>77</sup>

A series of 26 cases of *B. henselae* infection further elucidated its epidemiology, showing a preponderance of acquisition during the fall and winter.<sup>78</sup> In addition to previously described lymphadenitis, aseptic meningitis, recurrent chronic bacteremia, bacillary angiomatosis, and peliosis hepatitis, this report described newly recognized clinical presentations, including neuroretinitis, culture-proven lymphadenitis without the presence of antibodies to *B. henselae* (by immunofluorescence assay), and *B. henselae* bacteremia as a cause of chronic fatigue syndrome. Among HIV-infected patients, diseases included regional lymphadenitis, aseptic meningitis, and neuroretinitis, furthering the known clinical spectrum of *Bartonella* infections in this population.

Ocular disease occurs frequently among both immunocompetent and immunocompromised patients with *B. henselae* infections. This may present as unilateral conjunctivitis with associated regional adenopathy (Parinaud's oculoglandular syndrome) or as neuroretinitis, often with a macular star. Although spontaneous recovery has been documented in cases of CSD-associated neuroretinitis, the marked changes in visual acuity and ocular findings suggest the potential importance of antibiotic therapy for this condition. Reed et al.<sup>79</sup> studied seven cases of *B. henselae* neuroretinitis and their treatment. Alternative diagnoses such as HIV infection, syphilis, toxoplasmosis, and Lyme disease were excluded. Five of the seven patients

underwent fluorescein angiography, which showed papillitis, retinal edema, and increased capillary permeability. A regimen of doxycycline and rifampin for 4 to 6 weeks was administered, which shortened the course of disease and hastened visual recovery, compared with historical untreated cases. It was noted that the long-term prognosis is good, but some individuals may acquire a mild postinfectious optic neuropathy, with decreased contrast sensitivity and subtle changes in optic nerve function.

Throughout these investigations, antibiotic treatment regimens both before enrollment and after enrollment have been evaluated in the context of the growing body of literature regarding *B. henselae* therapy. Bass et al.<sup>80</sup> used a 5-day course of azithromycin in the treatment of CSD and demonstrated a significant clinical benefit, as measured by decreases in lymph node volume (measured by ultrasonography) within the first month of treatment, in a blinded manner. Col M.J. Dolan and colleagues recently published consensus-based treatment recommendations for the entire spectrum of diseases caused by *Bartonella* species.<sup>81,82</sup> The guidelines reflect the need for prolonged therapy for intracellular infections, especially in the setting of immunodeficiency, and the cumulative clinical experience, including retinitis treatment failures with macrolide monotherapy and success with combination therapy.

U.S. military researchers have contributed to other areas of *B. henselae* research, demonstrating that serostatus is not correlated with coronary artery disease<sup>83</sup> or neurocognitive decline in HIV infection.<sup>84</sup> Additionally, three state-of-the-art reviews have been completed.<sup>85-87</sup>

## Brucellosis

Brucellosis is a zoonotic disease of considerable economic import. Caused by *Brucella*, a genus of Gram-negative, coccobacillary, facultative, intracellular pathogens, veterinary infections primarily involve the reproductive tract of cattle, sheep, goats, and other animals, leading to septic abortion and orchitis, which can result in sterility. Four of the six known species (*Brucella abortus*, *Brucella melitensis*, *Brucella suis*, and, rarely, *Brucella canis*) are pathogenic in humans. Although brucellosis has a low mortality rate (5% of untreated cases), with rare deaths caused by endocarditis or meningitis, it is a relatively prolonged, incapacitating, disabling disease in its natural form.

## U.S. Military Significance

Infections among abattoir and laboratory workers suggest that brucellae are extraordinarily infectious via the aerosol route; it is estimated that inhalation of only 10 to 100 bacteria is sufficient to cause disease among humans. This has led some to consider the *Brucella* species as potentially incapacitating biological weapons. The relatively long and variable incubation period (5-60 days), coupled with the fact that many naturally occurring infections are asymptomatic, mitigates somewhat against the sinister use of *Brucella* species, although large aerosol doses may shorten the incubation period and increase the clinical attack rate. In fact, in its era of offensive biological warfare research in the 1950s, the United States chose *B. suis* as one of the first agents to be produced at the newly constructed Pine Bluff Arsenal in Arkansas.<sup>37</sup> Moreover, it is alleged that the South African Defense Forces, in the days of apartheid,



experimented with *B. melitensis* as a weapon.<sup>88</sup> Furthermore, Ken Alibek, the former deputy director of the Soviet biological weapons program, got his start in the bioweapons business by perfecting a culture medium for large-scale production of weaponizable brucellae.<sup>89</sup>

In addition to its potential importance as a biological weapon, brucellosis has a long history as an endemic threat on the battlefield. Thought to be the cause of "Crimean fever," which afflicted large numbers of British troops during the Crimean War, brucellosis also came to be known as "Malta fever" (*B. melitensis* infection, specifically) or "the Corps disease." Of note, Florence Nightingale was likely one of the most famous victims of Crimean fever during that conflict.<sup>90</sup> Brucellosis remained a significant threat to troops in the Mediterranean region and the Middle East during World War II. Large, persistent, enzootic foci among sheep and goats in the Middle East today, coupled with current large-scale U.S. military deployments to the region, make brucellosis a continued health risk to U.S. troops.

### U.S. Military Contributions

Much of the work on brucellosis conducted by military researchers has centered on vaccine development. Veterinary vaccines with significant efficacy against brucellosis have been studied and are widely used. As of this writing, the commercial animal husbandry industry has been declared "brucellosis-free" in all states except Texas and Missouri; vaccination is largely responsible for this success. The vaccination of livestock to reduce enzootic disease load, in combination with the slaughter of infected animals, is largely responsible for the declining incidence of human brucellosis. Most veterinary vaccines in use today are derived from *B. abortus* strain 19, an attenuated organism with stable virulence, or from Rev 1, a live, virulence-stable, *B. melitensis* strain. No licensed human vaccine against brucellosis is available in the United States, although live *Brucella* vaccines have been used in some nations in the past.<sup>91</sup> Typically, these preparations were derived from *B. abortus* strain 19, reflecting the cross-immunity among *Brucella* species and the diminished human virulence of *B. abortus*, compared with other species. Nonetheless, administration of either live preparation to humans is hampered by a notable incidence of clinical brucellosis cases, as well as by significant hypersensitivity reactions. Such problems were noted in the former Soviet Union, where human vaccination is still widely used, and in a U.S. trial of strain 19 and Rev 1 vaccines conducted 35 years ago.<sup>92</sup>

More recent attempts at vaccine development have examined fractional component preparations derived from various *Brucella* strains. Dr. David Hoover and colleagues in the *Brucella* Vaccine Development Program at the WRAIR used the outer membrane protein of *Neisseria meningitidis* as an adjuvant for intranasal *B. melitensis* immunization in mice and guinea pigs,<sup>93</sup> and a mucosal vaccine combining the outer membrane protein with purified lipopolysaccharide of *B. melitensis* is currently under study.

### Tularemia

Tularemia is a plague-like zoonotic illness caused by infection with *Francisella tularensis*, another Gram-negative coccobacillary organism. Many investigators recognize six somewhat dis-

tinct clinical syndromes caused by such infection, i.e., glandular (bubonic), ulceroglandular, oculoglandular, pharyngeal, typhoidal, and pneumonic tularemia. Two biotypes of *F. tularensis* are known; *F. tularensis tularensis* is found only in temperate areas of North America. Similar to the *Brucella* species, *F. tularensis tularensis* is an organism of extraordinarily high virulence, with as few as 10 cells representing the rabbit LD<sub>50</sub> and human 50% infectious dose.

### U.S. Military Significance

Because of its high virulence, tularemia (again, like brucellosis) was considered a potent biological weapon by both major superpowers during much of the Cold War. *F. tularensis palearctica*, however, is a low-virulence organism whose ecological niche appears to be in Europe and the former Soviet Union. The rabbit LD<sub>50</sub> of this organism is on the order of 10 million cells. For this reason, perhaps, the Soviets used an American *F. t. tularensis* strain, Schu-4, in their weapons program.<sup>88</sup> Interestingly, in what appears to have been an unusual example of Cold War cooperation, the strain was apparently obtained from U.S. scientists in exchange for strain 15, the isolate upon which Western vaccines are based. In 1955, the fledgling U.S. offensive biological warfare program selected *F. tularensis* to follow *B. suis* into weapons production at the Pine Bluff Arsenal.

In addition to the prominent position occupied by tularemia within the biological arsenals of the two Cold War superpowers, the disease has figured significantly as an endemic and epidemic wartime threat. During World War II, huge epidemics of tularemia affected tens of thousands of German and Soviet troops during various battles along the Don and Volga Rivers. Particularly noteworthy was the massive epidemic that occurred at Stalingrad from August 1942 to February 1943. This outbreak, which might have involved as many as 100,000 Soviet troops, began among German Panzer forces and then apparently spread to Soviet troops and citizens. This epidemiology, as well as an unusually high proportion (95.2%) of pneumonic cases,<sup>94</sup> led many, including Dr. Alibek,<sup>89</sup> to conclude that the outbreak originated with the intentional employment, by the Soviets, of weaponized tularemia. Others called this contention into question and concluded that the tularemia epidemic at Stalingrad was a natural consequence of wartime conditions and a breakdown in public health.<sup>95</sup> Whichever explanation holds true, it remains certain that tularemia significantly influenced military and medical operations during the battle and in its aftermath. Hundreds of cases of tularemia that occurred in postwar Kosovo in 1999–2000 further attest to the military relevance of this infectious disease.<sup>96</sup> Finally, sporadic outbreaks continue to periodically raise the possibility of bioterrorism<sup>97</sup> and make it likely that tularemia will remain both a military public health concern and a biowarfare/bioterrorism concern in the future.

### U.S. Military Contributions

As was the case with brucellosis, much of the work done on tularemia within the U.S. military has centered on vaccine development. Most of this vaccine work has involved the "live vaccine strain (LVS)" derived from Soviet strain 15. Despite the fact that the LVS-based vaccine is now 50 years old, no better product has surfaced, and investigators at USAMRIID, includ-

ing Dr. Dave Waag and colleagues, continue their work with this vaccine.<sup>98</sup> The Department of Defense Joint Vaccine Acquisition Program has taken on responsibility for shepherding the LVS-based vaccine through to Food and Drug Administration licensure.

## Conclusions

U.S. military medical services have made vital contributions to our understanding of bacterial zoonoses. Clearly, the most important contribution of the U.S. military medical services has been the development of a comprehensive research effort encompassing basic bacteriology, clinical research, vaccine development, and epidemiology to address these and unforeseen emerging diseases. Selecting the five most important specific research contributions is difficult; these include (1) advancing the understanding of *Y. pestis* virulence factors, pathogenesis, and ecology; (2) determining the genetic basis of *B. anthracis* virulence and clarifying the potential protective mechanism of anti-PA antibodies; (3) identifying subunit and/or attenuated vaccine candidates for plague, Q fever, brucellosis, and tularemia; (4) developing pre-exposure and postexposure immunoprophylaxis and chemoprophylaxis regimens for anthrax exposure; and (5) identifying etiological agents for CSD and defining the clinical spectrum of disease caused by *B. henselae*. In an era of emerging diseases and biological weapons, the U.S. military will continue to lead a dynamic research effort to counter these diseases.

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